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INNATE AND ADAPTIVE IMMUNE RESPONSES IN VIRAL AND CHRONIC INFLAMMATORY DISEASES

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It is quite possible to work without results,
but never will there be results without work.

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ABSTRACT

In this thesis I address some important questions regarding innate and adaptive immune responses in different human diseases. We analysed two distinct conditions: chronic autoimmune inflammation, specifically SLE, and chronic viral infection, represented by herpes simplex virus (HSV). The role of different components of the immune system were addressed, ranging from innate factors, such as IFNα and NK cells, to the adaptive immune system, represented by T-cells. The results are discussed with respect to the various possible levels of interaction between the innate and the adaptive immune systems.

We first focused on a specific subset of NK cells, $CD56^{bright}$ NK cells, which is normally found in lymphoid tissue and at sites of inflammation and which, upon activation, has the capacity to produce large amounts of cytokines. $CD56^{bright}$ NK cells are often discussed in relation to their possible role in shaping adaptive immune responses. The proportion of $CD56^{bright}$ NK cells was significantly increased in blood in subjects affected by SLE. This finding was not dependent on disease activity and may be due to increased levels of IFNα, a typical hallmark of SLE patients.

We then investigated several aspects of herpes virus infections. We describe a possible role for the activating NK cell receptor NKG2D in the immune response against HSV1 infection. We determined that HSV1 has the ability to downregulate the cell surface expression of NKG2D ligands of infected cells. We also observed that NK cells from patients affected by recurrent HSV1 manifestations have slightly increased levels of expression of NKG2D on blood NK cells during the acute phase of viral reactivation. In a prospective clinical study of patients with HSV genital infection, we observed that low specific T-cell responses against HSV antigens during primary HSV1 and 2 infection predicts a high frequency of clinical recurrences. Finally we examined the immune response of patients affected by recurrent meningitis caused by HSV2 infection. During asymptomatic periods, these patients showed elevated expression of TLR3 and TLR9, elevated IFNα production to certain stimuli and elevated specific T-cell responses when compared to patients with recurrent genital infection and to healthy seropositive donors. In addition, there were qualitative differences in their T-cell cytokine profile. We conclude that HSV2 meningitis is likely not a consequence of an impaired antiviral innate or adaptive immune response at the systemic level.

LIST OF PUBLICATIONS

Papers included in this thesis:

I. Danika Schepis, Iva Gunnarsson, Maija-Lena Eloranta, Jon Lampa, Stefan Jacobson, Klas Kärre, Louise Berg
Increased proportion of CD56^{bright} NK cells in active and inactive systemic lupus erythematosus
Immunology. 2009 Jan;126(1):140-6.

II. Danika Schepis, Mauro D'Amato, Marie Studahl, Tomas Bergström, Klas Kärre, Louise Berg
Herpes simplex virus infection downmodulates NKG2D ligand expression
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III. Elisabeth Franzén-Röhl, Danika Schepis, Fredrik Atterfelt, Kristina Franck, Arne Vikström, Jan-Åke Liljeqvist, Tomas Bergström, Elisabeth Aurelius Klas Kärre, Louise Berg, Hans Gaines
Herpes Simplex Virus Specific T-cell Response in Primary Infection Correlate Inversely with Frequency of Subsequent Recurrences
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Increased Cell Mediated Immune Responses in Patients with Recurrent Herpes Simplex Virus Type 2 Meningitis
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Paper not included in the thesis:

Kehmia Titanji, Francesca Chiodi, Rino Bellocco, Danika Schepis, Lyda Osorio, Chiara Tassandin, Giuseppe Tambussi, Sven Grutzmeier, Lucia Lopalco, Angelo De Milito
Primary HIV-1 infection sets the stage for important B lymphocyte dysfunctions
AIDS. 2005 Nov 18;19(17):1947-55.

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen-Presenting Cell
BCDA	Blood dendritic cell antigen
BCR	B cell receptor
CCR	C-C Chemokine Receptor
CD	Cluster of Differentiation
CMI	Cell-mediated immune
CNS	Central nervous system
CSF	Cerespinal fluid
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T-lymphocyte antigen
DC	Dendritic cell
DNAM	DNAX accessory molecule 1
ELISA	Enzyme-Linked Immuno Sorbent Assay
FACS	Fluorescence-activated cell sorter
GM-CSF	Granulocyte macrophage colony-stimulating factor
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSE	Herpes simplex type 1 encephalitis
HSV	Herpes Simplex Virus
ICP	Infected cell protein
IFN	Interferon
IL	Interleukin
ILT	Immunoglobulin-like transcripts
IP	Interferon gamma inducible protein
IRAK	IL-1R associated kinase
IRF	Interferon Regulator Factor
IS	Immunological synapse
KIR	Killer cell immunoglobulin-like receptor
LPS	Lipopolysaccharide

MBL	Mannose binding lectin
MHC	Major Histocompatibility Complex
MIC	MHC class I-chain related protein
MIP	Macrophage inflammatory protein
MMTV	Mouse Mammary Tumor Virus
MyD88	Myeloid differentiation factor 88
NCR	Natural cytotoxicity receptor
NFκB	Nuclear factor-kappa B
NK	Natural Killer
NKG	NK group
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen-Associated Molecular Patterns
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase Chain Reaction
PD	Programmed Death
PRR	Pattern Recognition Receptors
PVR	Polio virus receptor
RAET	Retinoic acid early transcript
RG	Recurrent genitalis
RM	Recurrent meningitis
RSV	Respiratory Cincitial Virus
SLE	Systemic Lupus Erythematosus
STAT	Signal transducer and activator of transcription
TAP	Transporter associated proteins
TCR	T-cell receptor
TGF	Transforming grow factor
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRIF	TIR domain-containing adaptor inducing IFN-ß
TYK	Tyrosine kinase
UL	Unique long segment
ULBP	UL16 binding proteins
Us	Unique short segmt
vhs	virion host shut-off

1 INTRODUCTION

1.1 IMMUNE SYSTEM

The immune system is a specialized network of organs, cells and soluble mediators that under normal circumstances maintain our body "immune" from diseases. In ancient time the word immune referred to people that were given political privileges; the word was then adopted to medical terminology with the meaning of protection from infection. Traditionally, the immune system is divided in two branches: innate and adaptive (for an historical review: Silverstein, A. M. 1989 *A history of immunology*. New York, Academic Press.)

1.1.1 Innate immune system

The innate immune system is present in all living eukaryote species and for this reason it is considered an evolutionary old form of defence. Its main characteristics are the limited diversity and the rapidity of action. It is normally defined as the first line of defence because it reacts immediately to outside pathogens without previous sensitization and before the adaptive immune system is able to mount an adequate response, which takes 4-7 days.

Leukocytes involved in innate functions have different roles: to engulf foreign agents in the body, to release cellular mediators such as cytokines and chemokines, and also to directly kill what is not recognized as foreign. The leukocytes of the innate immune system include macrophages, granulocytes, dendritic cells (DCs) and natural killer (NK) cells. The cellular component of the innate immune system is characterized by receptors, which recognize many related molecular structures called pathogen-associated molecular patterns (PAMPs) (1). These are molecular motifs consistently found on pathogens and not in the host. They are recognized by toll-like receptors (TLRs) (2) and other pattern recognition receptors (PRRs) (3), such as dectins and nucleotide-binding oligomerization domain containing (NOD), present in plants and animals. The PAMPs include several types of molecules, for example bacterial

Lipoposysaccharide (LPS), flagellin, lipoteichoic acid from Gram-positive bacteria, peptodoglycan and nucleic acid normally associated with viruses, such as double-stranded RNA (dsRNA) or unmethylated CpG motifs, among others. The recognition of these molecules leads to a rapid response of the innate system, and can result in a complete clearance of the pathogens or to its attenuation, in order to gain time for the adaptive immune system to act.

The role and function of DCs and NK cells in immune responses will be expanded in more detail below.

1.1.1.1 Dendritic cells (DCs)

Dendritic cells take the name from their surface projections similar to those of neurons (dendrites). They are resident in most tissues, including lymphoid organs, where they carry out their "sentinel" function: pathogens, as well as self-molecules, are normally engulfed through pinocytosis. C-type lectin receptors, such as DC-SIGN and TLR expressed on the cell surface, activate the DC to increase its pinocytoisis and processing of engulfed material. The ingested proteins are cleaved into peptide fragments that are then displayed at the cell-surface in association with MHC molecules. In fact, DCs belong to a specialized group of cells called antigen-presenting cells (APC). This stimulation enables DCs to migrate to the proximal lymph node. They also become activated and express co-stimulatory molecules, such as CD80 and CD86 (4) and increased MHC expression, which provide the second signal needed to activate T-cells. DC uptake and presentation of antigens from apoptotic cells, in the absence of a maturation signal, will instead induce T-cell tolerance. Activation of DCs also leads to local secretion of molecular mediators that can trigger endothelial activation and inflammation. Antigen presentation and cytokine release are the two major functions of DCs, and make these cells a perfect bridge between the innate and the adaptive immune response (fig.1).

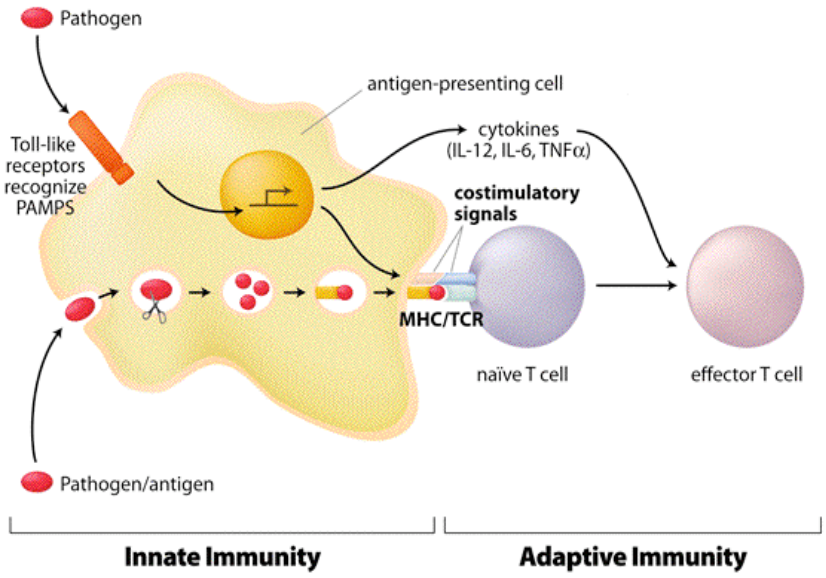

Fig1: From Innate immune responses to Adaptive immunity.
Antigen-presenting cells are activated through PRR recognition of pathogens. This activation leads to the production of cytokines and the expression of co-stimulatory molecules.
Antigens will be presented by MHC molecules to T lymphocytes (MHC/peptide/TCR trimolecular complex). This signal alone will induce tolerance on T-cells. To activate T lymphocytes, an additional signal from co-stimulatory molecules is needed. Activated T lymphocytes further differentiate to effector T lymphocytes. Figure modified from www.research.dfci.harvard.edu

It has become clear in recent years, that two different subsets of DCs exist: myeloid DC (mDC) and plasmacytoid DC (pDC). The developmental origin of mDC and pDC has been the debated in several publications over the last 10 years. The consensus is now that myeloid cells are able to generated mDC and lymphoid precursors will give rise to pDC (5). It seems that the presence of different transcription factors during DC ontogeny together with cytokines in the milieu will determine their final differentiation (6). DCs subsets can be distinguished by their surface markers as well as by their functions: both subsets lack lineage-specific markers, such as CD3, CD14 and CD19. They also lack CD56, are positive for MHC class II molecules and express a large variety of TLRs; furthermore, mDCs are $CD11c^{+}$ while pDCs are $CD123^{+}$ (IL3-Receptor α-chain, IL3Rα). In addition, pDCs in peripheral blood express BCDA-2 (7). One of the features that are relevant for the discussion in this thesis is the capability of pDCs to produce high quantities of interferon alpha (IFNα).

In 1999 Siegal et al (8) presented a sharper definition of the so-called interferon producing cells, already described in the 1978 by Trinchieri et al (9, 10), as pDC. These cells are the major source of type I interferon in response to viral infection, but also to immune complexes containing self-DNA from necrotic or apoptotic cells (11). pDCs secrete type I interferon upon ligation of specific TLRs that recognize RNA (TLR7 and 8) as well as DNA (TLR9) structure.

1.1.1.2 Toll like receptors

Toll like receptors were first discovered as involved in developmental biology by Nusslein-Volhard et al in 1980 (12). Their immunological role was identified in Drosophila where they can elicit antimicrobial response upon bacterial or fungal infection (13). Janeway et al identified the first human homologue and its involvement in immune response signalling, TLR4, in 1997(14). Since then, 10 human TLRs have been described (tab.1). They are mainly expressed on haematopoietic cells but also on fibroblasts and epithelial cells. They can be found on the cell surface (TLRs 1,2,4,5 and 6) or in the intracellular compartment, specifically within the endosome compartment (TLRs 3,7,8 and 9) (15). Once activated by their specific ligands, a cascade of downstream signals will lead to proinflammatory cytokine and chemokine production. The intracellular domains of TLRs, are generally coupled to an adaptor molecule called MyD88 (Myeloid differentiation factor 88) needed to transmit the signal to transcription factors such as NFκB (nuclear factor-kappa B) (16). Signalling through TLR3, TLR2 and TLR4 is also dependent on another adapter protein, TRIF (TIR domain-containing adaptor inducing IFN-ß).

Tab.1: Human Toll-like receptors and their ligands

NAME	LIGANDS	EXAMPLE
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
TLR2	Hemagglutinin protein peptidoglycan	Measles virus, HCMV, HSV1
TLR3	double-stranded RNA polyinosine-deoxycytidylic acid	Viruses synthetic compounds
TLR4	Envelope proteins LPS	RSV, MMTV Gram-negative bacteria
TLR5	Flagellin	Flagellated bacteria
TLR6	Zymosan Lipoproteins	Saccharomyces cerevisiae Bacteria
TLR7	single-stranded RNA imidazoquinoline	HSV small synthetic compounds
TLR8	single-stranded RNA imidazoquinoline	HSV R848
TLR9	DNA CpG-DNA	HSV1, HSV2, MCMV bacteria
TLR10	unknown	unknown

HCMV: Human cytomegalovirus; HSV: Herpes simplex virus; RVS: Respiratory sincitial virus; MMTV: Mouse mammary tumor virus; LPS: lipopolysaccharide

TLRs are expressed by different cell types and their activation can therefore translate into production of different sets of cytokines. For example, the production of IFNα by pDCs is favoured by the abundant and constitutive expression of IRF7 (interferon regulator factor 7) in pDC, a transcription factor downstream TLR7, 8 and 9 (17). TLR-mediated stimulation of monocytes and mDCs will lead instead to IL12 production (18). IL12, in cooperation with IFNα, in turn stimulates IFNγ production by NK and T-cells (19). IL12 is also necessary for CD8 T-cell and Th1 clonal expansion and full activation, being another link between innate and adaptive immune response. The importance of pDC-TLR-IFN signalling cascade in human diseases will be addressed later on.

1.1.1.3 Natural killer (NK) cells

In human, NK cells represent 5-15% of circulating lymphocytes. The NK cells are characterized by the absence of T-cell antigen receptor (TCR) and B cell receptor (BCR). Phenotypically they are defined by the combined lack of CD3 and presence of CD56 expression on their cell surface. The name "natural killer" originates from the initial notion that these cells can kill certain target cells and that they do not require activation or prior sensitization in order to do so, they are somewhat intrinsically “programmed” to kill. NK cells were placed into the innate arm of the immune system as they did not possess an antigen specific receptor, and were initially considered to be nonspecific in their interactions with target cells. However NK cells are far more complex than originally anticipated. NK cells have now been implicated for example in the control and clearance of malignant and virally infected cells, as well as in rejection of bone marrow transplants, in autoimmunity and in the maintenance of pregnancy (20). Thus it is clear that NK cells are more than simple killers, but have a multipotent role in the immune system.

It is possible to distinguish at least two major functional groups of NK cells according to their CD16 (FcγRIII) and CD56 expression: one group is $CD16^-$ and CD56 high, called $CD56^{bright}$ NK cells, and the second is $CD16^+$ and CD56 low, usually called $CD56^{dim}$ NK cells (fig.2).

CD56^{bright} NK cells are characterized by their ability to produce high amount of cytokines, such as IFNγ, TNFa and GM-CSF, making these cells relevant for immune regulation. CD56^{dim} NK cells instead, possess high killing ability due to the presence of lytic granules in their cytoplasm. This subset of NK cells is also capable of antibody-dependent-cellular cytotoxicity (ADCC) by ligation of their surface CD16 receptor in the interaction with antibody-coated target cells.

The two groups of NK cells show different organ distribution: CD56^{bright} cells are abundant in secondary lymphoid tissue, especially in the T-cell areas (21), while CD56^{dim} are located preferentially in peripheral blood and spleen (22). They also respond differently to IL2 stimulation due to a different distribution of the subunits of the IL2 receptor in the two NK subsets (23). In particular, CD56^{brigh} cells express the IL2 high affinity receptor, CD25 (24). Considering that most of the IL2 found in vivo comes from T-cells, this suggests that reciprocal regulatory mechanisms exist between NK and T-cells. It remains unclear if CD56^{bright} and CD56^{dim} NK cells derive from a common precursor (25) or if they represent different stages of NK cell maturation with different functions (26). However, experiments based on transfer of CD56^{bright} NK cells into mice lacking functional lymphoid cells suggest that CD56^{bright} NK cells represent an immature type of NK cell that can develop into CD56^{dim} NK cells (27).

Fig.2

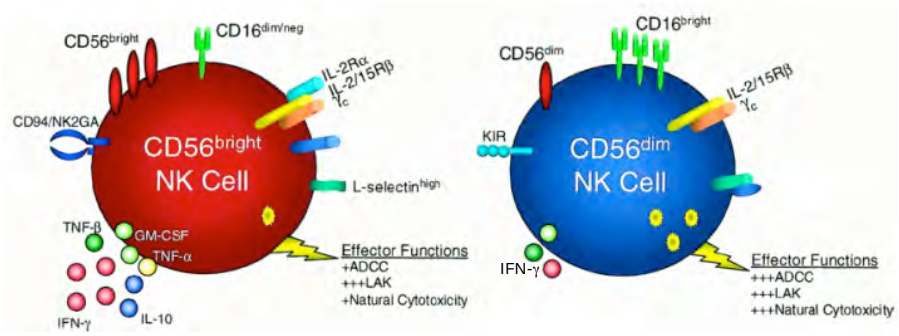

Fig2: Differences between CD56^{bright} and CD56^{dim} cells.
Adapted from Cooper MA, Blood 2001 May; 97(10):3146-3151

As shown in fig.2, CD56^{dim} NK cells are equipped with Killer Ig-like receptors (KIR). Both activating and inhibitory receptors are found within the KIR family

(tab.2). The concomitant presence of activating and inhibitory receptors on the same NK cell results in a tight regulation of activating and inhibitory signals (28). Stern et al initially observed that NK cells were able to kill tumor cells even if these lacked MHC class I molecules (29). This observation was subsequently developed in the "missing self" hypothesis (PhD thesis, Kärre K.,1981), suggesting for the first time the presence of inhibitory receptors on NK cells recognizing MHC class I, further developed later on (30). Since then, a variety of inhibitory and activating receptors have been identified (tab.2) also in other gene families a part from KIR (discussed below) but the understanding of the molecular mechanisms of NK cell activation and how the different signals are integrated is still incomplete.

A key contribution in this field has come from Bryceson et al Using an insect model they elucidated the essential minimal signals NK cells need in order to become activated (31). The consensus is that in normal physiological conditions, in absence of infected or tumor transformed cells, the inhibitory signals exerted through MHC class I recognition lead to inhibition of NK cell functions. Downregulation of MHC class I molecules on infected or transformed cells reduces the strength of inhibitory signals and therefore potentiates NK cell activation due to recognition, for example, of stress-induced ligands expressed on target cells. The activation of NK cells is translated into killing of the transformed cells through release of cytotoxic granules close to the contact site between the two cells (immunological synapse, IS). The lytic granules contain perforin and granzymes that lead to cell death through apoptosis (32). At the same time, NK cells rapidly secrete IFNγ that will promote antiviral and anti bacterial immune responses. IFNγ is also able to stimulate other cell types of the immune system such as macrophages, which will start producing proinflammatory cytokines (33), and CD4 T-cells, thus skewing the response toward a pro-inflammatory profile. NK cells constitutively express IFNγ mRNA that allow them to rapidly produce and secrete this cytokine (34).

In the late 90s a group of activating receptors unique to NK cells was identified: natural cytotoxicity receptors (NCRs, tab2). The NCR include: NKp30 and

NKp46, constitutively expressed by all NK cells (35, 36), and NKp44 that is normally expresses only upon activation (37). The natural ligands that these important activating receptors bind to, have been a mystery for a long time. Only recently it was published that they can recognize membrane associated extracellular matrix molecules, such as heparane sulfate proteoglycans (38). It seems that each NCR can recognize different microdomains on different heparane structures or haemagglutinin on virus infected cells (39, 40).
Another important activating receptor, NKG2D will be discussed in detail later on.

Direct NK cells cytotoxic and antiviral functions have been described since long, but recent data have shown that NK cells can also act as regulators of adaptive immunity, for example by interacting with and providing stimulatory signals to T-cells and dendritic cells (DCs). The role of NK cells in influencing adaptive immune responses and in the control of viral infection will be addressed later in the thesis in connection with paper I and II.

Tab2: A selection of NK cell receptors with their ligands

Receptor		**Ligand**
Killer Ig-like receptors (KIR)		
Inhibitory	*Activating* [a]	
KIR2DL1	KIR2DS1	Group 2 HLA-C Asn77Lys80 alleles
KIR2DL2	KIR2DS2	Group 1 HLA-C Ser77Asn80 alleles
KIR2DL3		Group 1 HLA-C Ser77Asn80 alleles
	KIR2DL4 [b]	HLA-G
	KIR2DS4	HLA-Cw4
KIR2DL5	KIR2DS5	Unknown
	KIR3DS1	Unknown
KIR3DL1		HLA-Bw4
KIR3DL2		HLA-A3, -A11
KIR3DL7		Unknown
Heterodimeric C-type Lectin receptors		
Inhibitory	*Activating*	
CD94/NKG2A	CD94/NKG2C	HLA-E
	CD94/NKG2E	Unknown
	NKG2D	MICA, MICB, ULBP-1-4, RAET1G
Natural cytotoxicity receptors (NCR)		
NKp30		Haemaglutinin, heparansulfate, BAT3
NKp46		Haemaglutinin, heparansulfate
NKp44		Haemaglutinin, heparansulfate
Immunoglobulin-like transcripts (ILT)		
ILT-1 (LIR-1)		HLA-G and other HLA molecules, CMV UL18
Activating receptors and co-receptors		
FcγRIII (CD16)		IgG
CD2		CD58 (LFA-3)
LFA-1		ICAM-1
2B4		CD48
NKp80		AICL
DNAM-1		CD112, CD155
CD69		Unknown
CD40 ligand		CD40

[a] Ligands for activating KIR are uncertain; [b] KIR2DL4 is functionally an activating KIR which mediates NK cell secretion of IFNγ, although it has ITIM motifs in its long cytoplasmatic tail. Modified from Farag SS, Blood review, 2006 May; 20(3):123-37.

1.1.2 Adaptive immune system

After a non-specific response from the innate immune system, adaptive immunity is required to generate a highly specific and efficient response to clear the pathogen. The adaptive immune system depends on B and T lymphocytes that express receptors of remarkable diversity, in order to effectively recognize pathogens and "antigens" of different nature. One characteristic of the adaptive immune system is that it can use a small number of genes to generate a vast number of different antigen receptors, which are then uniquely expressed on each individual lymphocyte. This is possible because of the so called V(D)J recombination process: a random rearrangement of antigen receptor gene segments. This recombination generates a diverse repertoire of T-cell receptor (TCR) and immunoglobulin B cell receptor (BCR). Moreover, somatic hypermutation contributes as an additional mechanism to generate an even higher diversity on BCR and secreted antibodies. Recognition of the specific antigen via BCR can directly induce B cell maturation into plasma cells with consequent specific antibody production, but this most often requires help from T-cells. T-cells instead can recognize antigens only in the form of peptides presented within the MHC complex.

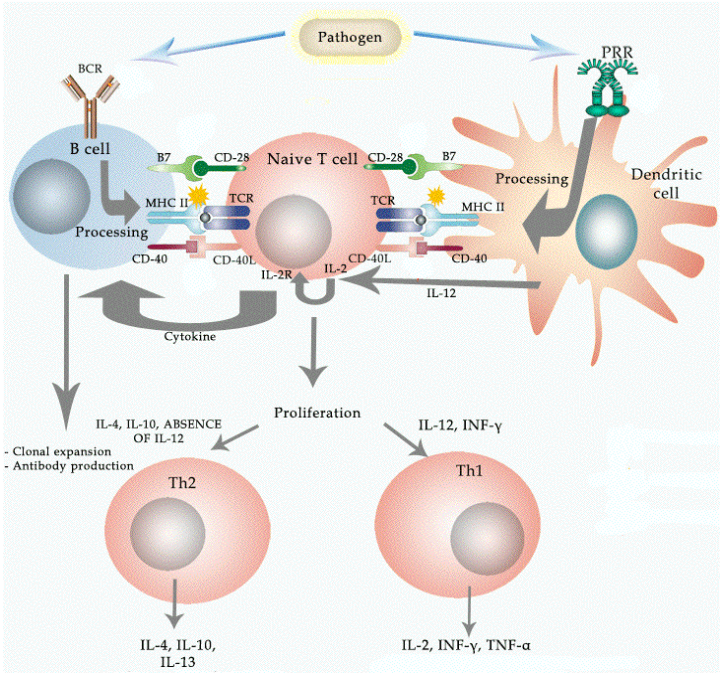

Fig.3: Schematic representation of signal integration between the innate and adaptive immune system. Pathogens can be recognized by: 1) PRRs on DCs. DCs will then present the antigen, together with co-stimulatory signals, to naïve T-cells that will proliferate and differentiate into Th1 or Th2 depending on the cytokines milieu. T-cells will also interact with B cells to induce their maturation; 2) BCR on B cells. B cells will mature and become antibody-producing cells. Adapted from: http://2008.igem.org

I will introduce in more detail the T-cell compartment, as this will be one of the topics of papers III and IV.

1.1.2.1 T cells

T cells represent the majority of circulating lymphocytes. They originate from the bone marrow and then migrate to the thymus where they undergo positive and negative selection to complete their development and then re-enter the bloodstream. These mature T-cells have not yet encountered their specific antigens and are called naïve T-cells. Naïve T-cells constantly circulate through secondary lymphoid tissue in search of their specific antigen. As described previously, APCs present antigens to T-cells when they meet in the lymphoid organ. For naïve T-cells, recognition and activation requires the presence of co-stimulatory signals. Once T-cells have been activated they start to produce IL2, whose main effect is to stimulate T-cell proliferation and clonal expansion.

Figure 3 shows a simplified view of T-cell activation where naïve T-cells after contact with APC, starts their process of differentiation. Before that, already in the thymus, T-cells have differentiated into $CD4^+$ and $CD8^+$ cells. Upon activation by APC in the periphery, $CD4^+$ cells can differentiate further into T helper (Th) 1 and 2 cells: Th1 mediate protective immunity to intracellular pathogens by promoting macrophage activation and neutralizing and opsonizing antibodies. They thus promote inflammatory responses and mediate the pathology in certain autoimmune diseases. In contrast, Th2 $CD4^+$ T-cells are considered to mediate protective immunity to extracellular pathogens and provide help for B-cell production of non-opsonizing antibodies. These cells and their cytokines are also believed to have anti-inflammatory capacity by controlling Th1 responses and viceversa. Th1 cells typically produce IL4, IL5, IL10 and IL13, while Th2 cells produce IL2, IFNγ and TNFα. In addition, within the $CD4^+$ T-cell subsets there are at least two additional groups of cells that need to be mentioned.

In the ’70s a T-cell subtype with suppressor capacity was proposed. These cells were termed T suppressor cells, but some irreproducible results caused the

research field to collapse, and the word was practically banned. In the '90, $CD4^+$ cells able to regulate immune responses could be more precisely defined and named T regulatory cells (T_{reg}) (41). New investigations of suppressive functions of T cells flourished. T_{regs} are able to suppress effector T-cell proliferation and cytokine production either through direct cell–cell contact or through the secretion of anti-inflammatory cytokine such as IL10 or TGFβ. There is also another important population belonging to the $CD8^+$ T-cell population with similar regulatory functions. $CD8^+$ T_{reg} are different in phenotype and function from the $CD4^+$ T_{reg} but have similar regulatory properties (42).

In 2003, a new subset of $CD4^+$ T-cells, sensitive to IL23 and important in controlling autoimmune reactions, was described (43). This new group of cells is able to secrete large amount of proinflammatory IL17, and they were thus named Th17 cells. Due to this characteristic, the role of Th17 cells has been investigated in the pathogenesis of several diseases with inflammatory features. In a recent review from Tesmer LA (44) it can be appreciated how the discovery of this specific subset of T-cells has been important for a better understanding of disease pathogenesis, for example in psoriasis as well as asthma and inflammatory bowel disease.

The $CD8^+$ T-cells, also called cytotoxic T lymphocytes (CTL), mainly exert a prominent cytotoxic activity. They are also able to secrete a variety of cytokines (45): once they recognize their antigen presented on MHC class I molecules they promptly release cytotoxic effector molecules such as perforin (46), granzymes, granulysin and also start expressing surface bound Fas ligand (47) with consequent death of the target cells. These mediators have no specificity of recognition, thus their release has to be tightly controlled. The activation pathway resembles that of $CD4^+$ T-cells in the way that they also need co-stimulatory signals in order to be activated from the naïve state, but the level of co-stimulatory signals is higher than the one needed to activate $CD4^+$ T-cells. There is evidence that $CD8^+$ T-cell interaction with the proper APC is determined by a previous contact of the very same APC with $CD4^+$ T-cells (48), even though this concept is still under debate. Chemokines released during this interaction

will attract $CD8^+$ T-cells and this will increase the likelihood of specific $CD8^+$ T-cells interacting with this very APC, which has been identified by $CD4^+$ T-cells to express foreign antigen. Naïve $CD8^+$ T-cell clones expand significantly more upon antigen stimulation compared to $CD4^+$ T-cell clones, and $CD8^+$ clones generally represent a much larger population of effector cells (49).

After activation, negative regulation mechanisms start to take place in order to avoid a prolonged T-cell response, which would lead to excessive tissue destruction and possibly autoimmunity. In this contraction phase the majority of the reactive T-cells will undergo programmed cell death. Surface receptors with suppressive property, such as for example cytotoxic T-lymphocyte antigen 4 (CTLA4) or Programmed Death 1 (PD1), carries this out. The absence of specific antigens and cytokines will also decrease T-cells activation. The majority of activated $CD4^+$ T-cells die after antigen clearance, but a small number turns into a resting state and becomes memory cells. As described above, signals from the TCR following recognition of peptide–MHC complexes determine T-cell development, survival and death, as well as subset lineage commitment and differentiation into effector or memory T-cells. The strength of this signal, which depends on affinity between TCR and MHC-peptide complex, and the concentration of TCR-peptide–MHC complexe interactions, are some of the factors influencing the different outcomes. It was shown in 1997 that when T-cells interact with DCs they receive a signal that will induce T-cell adherence on DCs and subsequent activation (50). After this first observation, a lot of data on DC-T interaction have been produced, thanks also to new imaging technologies. Recently, it has been reported that the stability of DC-T-cells contact depends on the number of specific MHC-peptide complexes presented by each DCs (51). It seems clear that longer contact facilitates T-cell activation, while it is not clear whether the time of interaction depends on DC level of maturation or T-cell state of pre-activation, or if it is just a random event (52).

Both $CD4^+$ and $CD8^+$ T-cells can differentiate into memory cells. Several observations support the concept that memory T-cells can be generated during resolution of infection as well as throughout the course of an immune response

(53). Memory $CD8^+$ T-cell numbers are generally quite stable over time after antigen/pathogen clearance, while memory $CD4^+$ T-cell numbers often decline. This has been observed in many different experimental systems including acute and chronic viral infections (54, 55) or bacterial infections (56). An important definition made by Sallusto et al has been the phenotypic differentiation between central memory cells, expressing the CCR7 chemokine receptor, and effector memory cells, lacking CCR7 (57, 58). Both memory compartments express CD45RO, an isoform of a CD45 protein tyrosine phosphatase, but not CD45RA. Central memory cells represent a population resident in lymphoid organs that have a high proliferative potential and can efficiently stimulate dendritic cells and generate a new wave of effector cells following antigen re-encounter. On the contrary, effector memory cells represent a population of T-cells that are prone to secrete high amounts of cytokine, in particular IFNγ, and can rapidly mediate effector functions following pathogen challenge, due to a lower threshold of activation for TCR signaling (59).

Lately, a fixed lineage model has been introduced describing that the memory T-cell compartment might arise directly from a precursor that diverges early during an immune reaction recognizable by the high expression of IL7 receptor (60), rather than being a subset of T-cells that develop as a consequence of effector/antigen exposed T-cell.

1.2 IMMUNE SYSTEM IN DISEASES

1.2.1 Autoimmunity

Autoimmunity is caused by immune reactions of an organism against its own cells or tissues. To avoid this, the body's immune responses are subjected to several checkpoints, where potentially autoreactive cells are eliminated or made inactive. For instance, more than half of all antigen receptors randomly generated by V(D)J recombination recognize self-antigens (61-63), with potential detrimental consequences for the organism itself. Checkpoints exist at multiple

locations: 1) in the thymus, where T-cells with strong TCR binding to self antigens are eliminated via apoptosis; 2) in peripheral lymphoid organs, where recognition of self antigens by naïve T-cells through TCR alone is inefficient to trigger activation, and usually results in quiescence (anergy); 3) in target tissues, where inhibitory signals contribute to dampen T-cell activation: prolonged stimulation of the TCR induces feedback mechanisms that further limit the potential for excessive growth and activation, like for example those generated via CTLA4 (64); 4) the tight control exerted by the T_{reg} population.

Autoimmunity is a result of many different factors. Even though the body is equipped with control mechanisms that prevent immune reactions against self-antigens, autoimmune diseases can still develop. Risk factors can for example be inherited defects in the genes coding for proteins involved in such "checkpoint" functions and/or to environmental factors that can trigger the immune response and progression.

I will discuss one specific autoimmune disease a little bit more in detail, the subject of paper I.

1.2.1.1 Systemic Lupus Erythematosus (SLE)

SLE is a heterogeneous autoimmune disease characterized by chronic inflammation and tissue damage in various organs. Patients with lupus present abnormal autoantibodies in their bloodstream, which are often specific for nucleic antigens. Because these antigens can be found anywhere in the body, lupus has the potential to affect a variety of areas: skin, heart, lungs, kidneys, joints, blood vessels and the nervous system. The disease is characterized by periods of illness, flares, and periods of remission. From studies of identical twins and familial cases, it is clear that genetic factors play a role in SLE (65) with a disease concordance in identical twins of around 35%. A peculiar observation is that SLE is nine times more common in women than in men, especially during female hormonal cycle, implying a hormonal component in the pathology of the disease (66). One of the processes, which are believed to play a role in the

pathogenesis of SLE, is apoptosis. Mouse strains defect in the process of apoptosis develop lupus like disease (67, 68). Furthermore, failure in clearance of apoptotic material, due for example to defects in macrophage uptake of apoptotic cells (69) or to defects or mutations in the complement pathway and DNAse are associated with SLE disease (70, 71) . Thus, high amounts of nuclear autoantigens are generated as a consequence of deficient apoptosis and clearance of immune complexes. These nuclear fragments, in complex with auto-antibodies, are recognised through Fc receptors on DCs. They are internalized, and presented on MHC to T-cells that are not tolerized to nuclear antigens since they are normally not presented in the thymus. More over, abnormalities in PD1 (72) and CTLA4 inhibiton of T-cell activation can induce lupus like disease.

Elevated IFNα production is observed in SLE. Increased serum level of IFNα correlates with both frequency and severity of disease relapses (73). IFNα, originally described in the context of its antiviral function, can cause excessive inflammation and thus induce tissue damage. The first link between this chronic inflammatory disease and IFNα was based on two observations: 1) in a patient receiving IFNα as therapy to treat malignant carcinoma, clear signs of SLE hallmarks appeared (74) and, 2) increased levels of IFNα was observed in sera of patients suffering from SLE compared to healthy control (75). Later the presence of pDCs in skin lesions of SLE patients has also been documented (76). Today, the expression of type 1 interferon genes and interferon induced genes are considered a 'signature' in SLE disease (77, 78). The abundance of IFNα in the blood stream of SLE patients can lead to the maturation of monocytes into DCs capable of presenting auto-antigens, derived for example from dying cells, to autoreactive $CD4^+$ T-cells (79). At the same time, IFNα production by pDCs can directly induce B cell maturation into antibody-producing plasma cells (80). In SLE, there is an abundance of autoantibodies specific to nuclear antigens, which can form immune complexes. These immune complexes can bind and stimulate pDCs to produce high levels of IFNα (81), possibly through interaction between nucleic acids present in the immune complexes and intracellular TLR (82). In line with this, treatment of lupus-prone mice with TLR7 and 9 antagonists results in a reduction of disease severity (83). In this way, pDC-TLRs-IFN pathway not only is

crucial in providing an appropriate response to infections, but can also trigger mechanisms that lead to chronic autoimmune inflammation (fig.4).

Fig.4

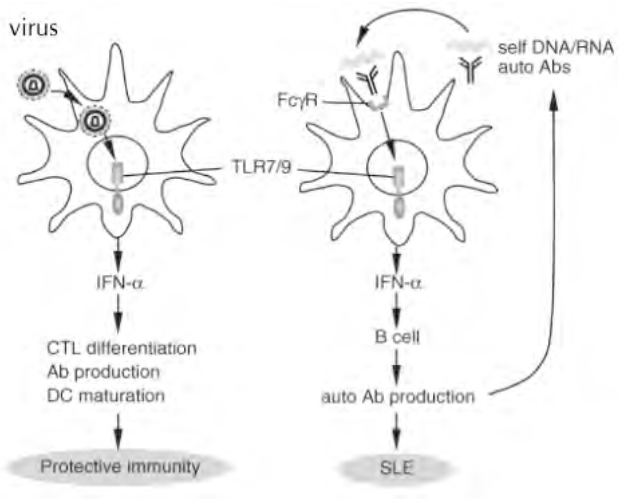

Fig.4: Scheme of pDC response to different stimulations.
To the left: After viral infection, pDC derived IFNα promotes immune activation. To the right: IFNα can participate in the pathogenesis of SLE by promoting maturation of autoantibody-producing cells generating a pathogenic loop.
Adapted from Ohteki T, Allergology International 2007 Sep;56(3):209-14

Do NK cells have a role in SLE? While the role of NK cells was initially described primarily in the context of tumor surveillance and viral immunity, substantial evidence has accumulated for their contribution to autoimmunity (84). However, reports are still somewhat contradictory: animal studies suggest that NK cells can either enhance or limit adaptive immune responses and inflammation, thus having a protective or promoting role in autoimmunity, depending also on disease stage (85). This difference may be explained by the fact that NK cells are able to produce different cytokines, including: 1) IFNγ which acts on macrophages suppressing Th2 and promoting Th1 responses, and 2) IL13, which inhibits the production of inflammatory cytokines, and promotes a Th2 response (86).

In SLE patients a reduced number of circulating NK cells and defects in their killing capacity has been observed (87, 88). It is not clear if the described change in the NK cell compartment represents a primary NK cell defect, which could play a pathogenic role, or if this is a secondary effect due to the disease itself or to the treatment of disease. A variant of the Fc receptor CD16 with lower binding capacity, thus resulting in diminished NK cell ADCC function (89), has been associated to SLE disease with possible important consequences in SLE

pathogenesis. Considering also that cytokine-producing $CD56^{brigh}$ NK cells have the capacity to promote T-cell activation in lymphoid organs, which may result in B cell activation, it is possible that they can, directly or indirectly, participate to the pathogenesis of the disease.

It is clear that factors modulating NK cell number and response may be implicated in the pathogenesis of autoimmunity but further studies are needed to clarify the mechanisms involved.

1.2.2 Anti viral immunity

The immune system, both the innate and adaptive compartments, has evolved together with a large variety of viruses. One can regard many viral infections as being well tolerated and cleared without life-threatening complication due to a well-balanced co-evolution of host-microbe interactions. For example, in latent viral infections, such as that of members of the herpes family, the balance appears optimal for both virus and host although the infection can have fatal consequences when the normal immune system is perturbed. One of the principal immunological mechanisms identified to fight viral infection has been the IFN system. IFNs were discovered more than 50 years ago by means of their antiviral activity and the capacity to "interfere" with viral replication (90-92). The IFN family can be divided into three subfamily: type I (IFNα, β, ω, κ and τ), type II (IFNγ) and the more recently described type III (IFNλ). In humans there are at least 13 genes encoding IFNα and together with the other members of the type I IFN, the proteins encoded by these genes interact with the very same receptor. One can imagine that such a multitude of genes may provide the necessary flexibility to control the different biological activities that this group of molecules has: antiviral functions, antiproliferative activities, cellular differentiation, inflammation etc (93-95). Discussing the different proteins included in the IFNs family is beyond the scope of this thesis, and the remaining discussion will thus focus on the importance of IFNα to lead the reader through an easier comprehension of my thesis.

1.2.2.1 IFNα and viral infection

An important function of IFNα is to induce a state of resistance to viral replication in all cells. Once it has been produced by virally infected cells, type I IFN will be recognized by its receptor on neighbouring cells, and this will induce production of proteins that help to inhibit viral replication and thus viral spread. IFNα also has the potential to increase the expression of MHC class I molecules on different cell types. In this way IFN will indirectly facilitate the killing of infected cells by cytotoxic T-cells that recognize complexes of viral antigens presented by the MHC class I molecules. Moreover, the genes responsible for IFNα production are themselves induced by IFNα, resulting in a positive feedback loop that can amplify the innate response to viruses. Impairment of the IFNα signal cascade will result in deficient antiviral and antibacterial responses. In humans, two children have been described with mutations in the molecule downstream the interferon receptor, STAT-1 (signal transducer and activator of transcription 1). This mutation has been reported to be correlated with increased susceptibility to mycobacterial and viral infection (96) with potential fatal consequences. Both of the described children died from viral illness, and in one herpes simplex type 1 (HSV1) encephalitis (HSE) could be confirmed. In another report, a deficiency in one of the kinases associated with the interferon receptor, TYK2 (tyrosine kinase 2), was documented in a child suffering from recurrent cutaneous infection caused by HSV1 with highly impaired cytokine response (97). IFNα can indirectly induce IFNγ production by stimulation of T and NK cells. However, IFNγ specific defects are not associated with disseminated viral infection (98).

There are also defects upstream IFNα production that have been reported to couple with several kinds of bacterial and viral infections (99). The majority of data available today come from animal models. A few rare human cases are also known, which highlighted the importance of different molecules involved in the IFNα pathway. The first to be described was the IRAK-4 deficiency (IL-1R associated kinase 4) (100). This molecule associates with MyD88 downstream TLRs activation. Patients with a mutation in IRAK4 present reduced IFNα production in response to TLR7,8 and 9 agonists, have disseminated bacterial

infections, but are able to cope with several common viral infections (101), suggesting that the IRAK4 signal is not essential for viral immunity. In 2006 Casrouge et al. (102) discovered a mutation in the UNC-93B1 gene that leads to a complete loss of expression of the corresponding protein. This protein is essential for the translocation of TLR 7,8 and 9 from the endoplasmic reticulum to the endosomes. Without UNC-93B, TLRs response to ssRNA or dsDNA is abrogated and this results in a loss of IFNs production. Patients affected by this mutation suffer from Herpes simplex type 1 encephalitis but can clear other viral infections, suggesting a specific role for UNC-93B in controlling the immunity to HSV1 in the central nervous system (CSN). One year later, TLR3 mutations were reported to associate with low IFNs production and susceptibility to Herpes simplex type 1 encephalitis (103). The evidence that these mutations correlate only with Herpes simplex type 1 encephalitis implies that there may be redundant molecules that have an important role in providing protection against other viral infections, while in Herpes simplex type 1 encephalitis, TLR signalling and IFN production is vital. Certain gene polymorphism can also directly affect the predisposition to viral infection. A report in 2007 (104) showed that TLR2 polymorphisms are associated with increased frequencies of HSV2 genital recurrences and periods of viral shedding. Altogether, these studies pinpoint the importance of an intact and functional TLRs-IFNα signal pathway in the triggering of appropriate immune responses against infections and, in some cases, specifically in infection caused by herpes simplex virus.

1.2.2.2 NK cells and viral infection

The first evidence for an interaction between NK cytotoxicity and viral infection came from an in vitro study where Santoli et al. (105) showed that IFNγ stimulation of NK cells potentiates the cyototoxic activity against infected cells. Confirmations arrived later with NK cell depletion studies in mice (106) and with the observation of naturally occurring NK cell deficiencies in humans (107). The roles of NK cells in different viral infections are several and very complex (108), but for the purpose of my thesis, I will review some of the latest findings about

NK cells in human viral infections and focus more specifically on herpes infections.

Studies of viral infection caused by influenza virus have identified ligands for the Natural cytotoxicity receptors (NCRs). In 2001 Mandelboim et al (40) identified viral hemagluttinin as a ligand for the NKp46 receptor, while another group discovered that the very same ligand is also recognized by NKp44 (109). A recent study has shown that even though NK cells are able to recognize influenza infected cells through NCR (110), in the first hours after infection NK killing capacity is reduced due to a reorganization of inhibition signals in the lipid rafts on the cell surface of infected cells. This reorganization probably causes clusters of MHC class I proteins, which increase NK cell inhibition (111).

A role for NK cells has also been suggested in hepatitis C infection. During chronic infection expression of NKp30 and NKp46 is upregulated, together with increased NK cell production of IL10 (112). Contradicting data on dowregulated levels of NCR exist (113) with paralleled decreased NK activity. These and other changes in NK cell phenotype during chronic HCV infection (114) imply either that NK cells are involved in the pathogenesis or affected by the disease. A recent in vitro study reports an antiviral effect of NK cells in HCV infection due to the induction of IFNα and IFNα induced genes (115). Another type of evidence for a role of NK cells in viral diseases comes from an interesting genetic aspect of NK cell biology: the extreme interindividual variation in the expression of KIR receptors (tab.2). The number of KIR genes and their expressed alleles can differ vastly from one individual to another. This characteristic led to epidemiological studies aimed at assessing the impact of KIR and HLA variability in human diseases. In hepatitis C infection a direct association between a given combination of KIR and HLA ligands and protection from HCV infection has been reported (116). Since then, other studies followed showing KIR genetic associations in HCV, specifically a beneficial effect of having the inhibitory KIR2DL3. This specific KIR has the lowest binding affinity to HLA-C among the KIR, possibly increasing the probability of activating KIR binding to HLA-C (117).

Studies on NK-KIR have been performed during the course of another chronic infection, human immunodeficiency virus (HIV) (118). The involvement of NK cells during this viral infection has been studied since 1986 (119), when a reduction of circulating NK cells was observed. This reduction seems to be partially due to the emergence of a novel subset of NK cells, which are rare in healthy individuals; the $CD56^{-}CD16^{+}$ NK cells (120, 121). These $CD56^{-}$ NK cells lack the majority of NK cell effector functions, including killing, cytokine secretion, antibody-dependent cellular cytotoxicity (ADCC), and exhibit defects in DC editing activity (122). Later on, strategies of HIV to avoid NK cell recognition has been studied (123): HIV infected cells were able to escape NK killing and this ability was dependent on retention of HLA-C and E on infected cells (124), while HLA-A and B are down modulated to avoid T-cell recognition. More over an epistatic effect of HLA-Bw4 and a specific KIR, KIR3DS1 has been described in protection against disease progression in HIV (125) even though the mechanism has yet to be clarified. Another important finding is the observation that in exposed but non-infected subjects, NK cell activity was augmented (126), suggesting a role for NK cells in early clearance of the virus. In general, genetic studies associating KIR genes and alleles to risk to develop disease or ability to clear viral infections is no direct proof of NK cell involvement for at least two reasons. KIR molecules are expressed not only by NK cells, but also by T cells. In addition, genetic associations may be due to linkage of other genes to the gene under observation.

There are also several case reports that indicate a role for NK cells during herpes virus infection (127-129). In 1985, Fitzgerald et al. showed that NK cells are important in the protection against murine HSV1 by controlling viral replication mainly through the production of IFNγ (130). Mice deficient in IFNγ are susceptible to the development of cutaneous zosteriform lesions (131) and IFNγ has been shown to be important in controlling viral reactivation. Mouse studies have also shown the importance of NK cells during HSV2 infection (132). Part of the protective function of NK cells in HSV infection is believed to be due to the virus ability to down regulate MHC class I surface expression (133). Very likely, the virus has evolved this mechanism in order to avoid T-cell recognition of

infected cells. It is also described that NK cells are able to kill cells infected with HSV1 (134-136). The importance of NK cells during herpetic infection has been extensively described for cytomegalovirus (CMV), a member of the herpes viruses. Particular interest has been focused on the ability of this virus to affect the expression of NKG2D ligands in infected cells (28, 137). I will dedicate some extra words to describe this receptor to give a more complete introduction to paper II.

NKG2D (NK group 2 member D) is an activating NK receptor belonging to the c-type lectin like family (tab2). It is expressed also by $CD8^+$ and γδT-cells. Expression of NKG2D has been reported also on $CD4^+$ T-cells, but only during chronic inflammation (138, 139). NKG2D on T-cells acts as a co-stimulatory receptor, rather than a primary activating receptor. Its expression is regulated by cytokines, in particular IL15 improves NKG2D expression (140, 141) while TGFβ and IL21 dowregulate it (142, 143). NKG2D binds MHC class I related proteins (MIC) A and B and UL16 binding proteins (ULBPs 1-4) which are expressed during viral infection and tumor transformation (144), but rarely in healthy cells. The expression of NKG2D ligands is tightly regulated by activation of transcription factors and also at the post-transciptional level by microRNAs (145), ubiquitination (146) or cleavage by metalloproteinases at the cell surface (147). The important role of NKG2D during viral recognition is highlighted by the various mechanisms that different viruses have evolved to avoid NKG2D recognition (148). For instance: human CMV (HCMV) produces two proteins, UL142 and UL16, which dowregulate and sequester several NKG2D-ligands in the endoplasmatic reticulum (149-152).

1.2.2.3 T-cells and viral infection

As already noted, innate immune responses can be sufficient to eliminate pathogenic agents, but an appropriate adaptive immune response is often needed. Several viruses, including HIV, HCV and herpes viruses are able to escape immune control and establish chronic infection. One of the characteristics that these viruses share is their ability to elude virus-specific T-cell

responses (153)

In the case of HIV, the virus directly infects and depletes $CD4^+$ T-cells. This, as well as other virus-induced immune modulatory mechanisms not well understood, leads to an impairment of the adaptive immune response of the host and eventually to an immune-compromised state. During the progression of the disease there is also impairment of HIV-specific $CD8^+$ T-cell responses: cells present with normal proliferative and cytokine responses but impaired killing capabilities (154, 155).

During chronic HCV infection the T-cell response is impaired at different levels including proliferation and cytokine production (156). Lack of immune control during chronic HCV infection might be due to viral mutations or can be due to incomplete differentiation of effector and/or memory T-cell populations. It can also be that there is an immune exhaustion resulting from persistent high viral load. On the contrary, during acute infection, sustained activation of HCV-specific T-cells is associated with viral control (157). Protection from persistent HCV infection is dependent on both $CD4^+$ (158) and $CD8^+$ (159) T-cells. During acute infection, for example, $CD8^+$ T-cells, in the blood and liver, display IFNγ production and cytotoxic activity in response to a huge variety of HCV peptides (160), while the loss of early $CD4^+$ T-cell responses predicts recurrence of viremia and development of persistent infection (157).

The acquired immune response to herpes viruses includes both $CD4^+$ and $CD8^+$ T-cell activation. $CD4^+$ T-cells play a crucial role in coordinating the immune response in the initial phase of infection and, in fact, $CD8^+$ T-cells develop poorly in absence of specific $CD4^+$ T-cells. It is also evident that herpes infection, possibly by reduction of CD83, influences DC priming of T-cells, affecting indirectly the pool of effector cells (161). For example in a mouse model of genital HSV2 infection, the first immune response in the epithelium is mediated by recruited DCs that then migrate to the draining lymph node to present HSV2 antigens to $CD4^+$ T-cells (162) which will start to produce IFNγ. Moreover, memory $CD4^+$ T-cells orchestrates the local antiviral response (163). The local immune responses seem to be controlled by the action of $CD4^+T_{reg}$ cells that

facilitate cell migration into infected tissue (164). On the other side, $CD8^+$ T-cells are central in controlling herpes latency. They have been detected in trigeminal ganglia of HSV1 infected patients (165) where they selectively suppress viral reactivation by production of IFNγ and non-cytotoxic granules (fig.5) (166-169). In herpes infection NK cells are believed to contribute to the early control of the virus, while $CD8^+$ T-cells are essential for the control of the infection, as well as in control of reactivation in latent infection. It may be that a strong initial NK cell response reduces infection of DC and hence priming of $CD8^+$ T-cells, thus prolonging the virus productive phase. Conversely, if NK cell response is weak this may facilitate the generation of specific $CD8^+$ T-cell responses, accelerating the establishment of latency.

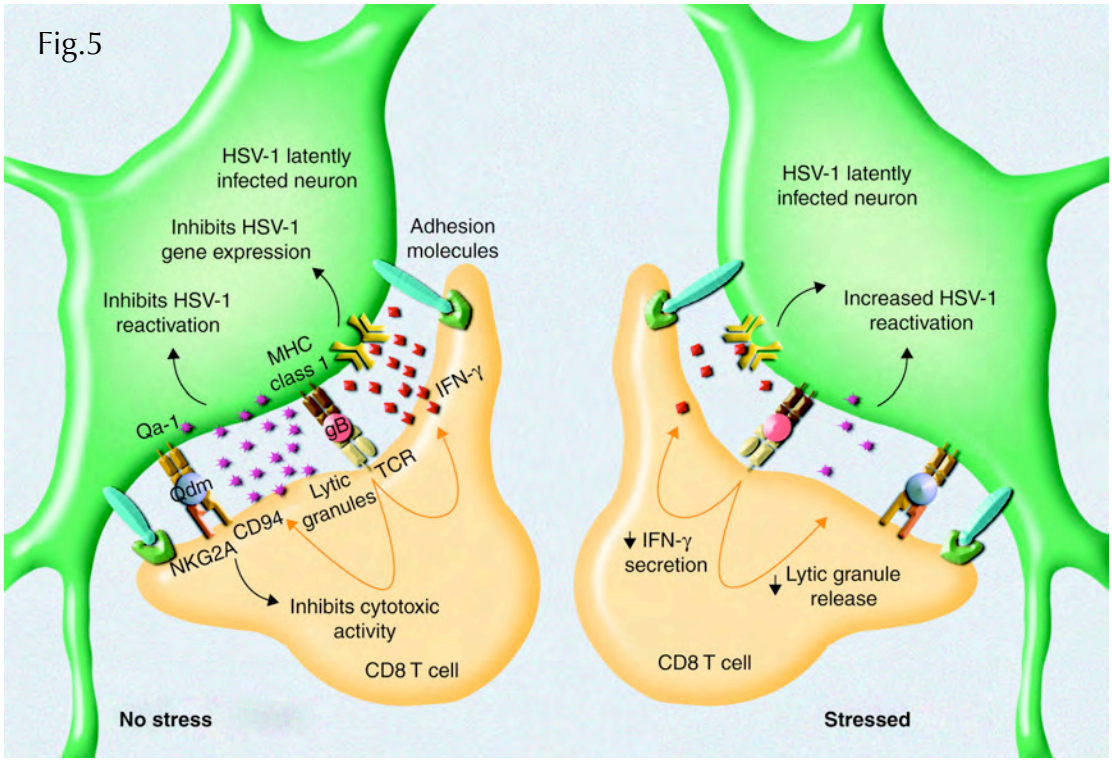

Fig.5: Suggested mechanism used by $CD8^+$ T-cells to inhibit HSV1 reactivation from ganglia. $CD8^+$ T-cells use IFNγ and non-cytotoxic lytic granules to inhibit HSV1 reactivation from latency. Under stress CD8 T-cells secrete reduced amounts of IFNγ and lytic granules, which results in protein and viral reactivation within infected neurons.
Adapted from: Sheridan B.S., Expert Opinion on Biological Therapy, 2007 Sep;7(9):1323-31

1.3 HERPES VIRUSES

1.3.1 Herpes simplex virus type 1 and 2

HSV1 and HSV2 are large DNA viruses that belong to the alphaherpesvirinae subfamily, classified based on their relatively short reproductive cycle, a variable host range, an efficient destruction of infected cells and their ability to establish latent infections primarily in sensory ganglia. Herpes simplex viruses are particularly interesting because of the variety in clinical symptoms elicited in different individuals, ranging from non-symptomatic infections to recurrent genital/oral blisters or neurological complications. I will come back to the wide variety of symptoms and what they may be due to in the discussion of paper III and IV. Here, I will concentrate on the virus itself.

HSV1 and HSV2 genomes are quite similar and contain at least 84 transcriptional units that encode proteins. The genes are classified based on their time of transcription: α or immediate early, β or early and γ or late. The transcription is sequential: the transcription of one class determines the transcription of the other. To initiate infection, the virus has to come in contact with the host cell. Fusion of the two membranes takes place and the virus releases its genome into the cell. This is then transported into the cell nucleus where replication of new viral proteins can take place. The virus in its productive phase uses the host RNA polymerase.

1.3.1.1 Interference with host immune response

One of the characteristics of the herpes viruses is the ability to produce several proteins that affect the host cellular defense. Examples for this are the virion host shut-off (vhs) protein which degrades eukaryotic mRNA (170), and the ICP27 protein, which blocks splicing of mRNA (171). These virally encoded proteins dampen the cell's ability to produce its own proteins while this will facilitate the production of viral proteins. Moreover, to achieve the full replication cycle the virus has to keep the infected cell alive. Viral Us3 and ICP6 are able to block the

apoptotic process otherwise dangerous for the virus's ability to propagate. Importantly, herpes virus proteins also target specific pathways involved in the host immune response to infections. Two proteins have been identified that influence directly IFN production in infected cells: ICP0 (172) and ICP34.5 (173). However, virus-driven impairment of IFN signalling is not as dramatic as in the rare human mutation described for STAT1, which causes fatal HSV infection (96). This indicates that a complete shut-off of the IFN pathway is harmful to the virus, which has co-evolved with the host by developing suitable ways to maintain an equilibrium that preserves both organisms.

More recently, the HSV protein ICP0 has been identified as an attenuator of TLR signaling, hence inhibiting innate responses to HSV (174). In addition, in a publication from Chisholm et al (136) this protein was shown to be sufficient for NK cell recognition and killing of infected cells. It was proposed that ICP0 acts as a ligand for NCRs, thereby activating innate immune responses to HSV. The role of ICP0 in influencing the immune response to HSV is thus somewhat contradictory.

One of the fist immune evasion mechanisms described for HSV was the dowregulation of MHC class I molecules due to the interaction of ICP47 with transporter associated proteins (TAP). The interaction blocks the TAP dependent transport of peptides from the cytoplasm into the endoplasmatic reticulum, thus diminishing MHC class I surface expression. In this manner, HSV suppresses $CD8^+$ T-cell recognition of infected cells (175). In 2001 the product of the Us1 gene was reported to inhibit B cells induction of $CD4^+$ T-cell activation (176).
Although mechanisms by which HSV1 and HSV2 avoid immune surveillance are being revealed, a major issue in clinical prevention is what determines HSV severity and recurrence frequency. There are a few reports on HSV recurrences or symptomatic vs asymptomatic infection and HLA association (177, 178) pointing towards T-cells as major contributors to the disparate clinical manifestations. Also, a more recent paper associates KIR genes with HSV1 disease course (179), which could be attributed to T-cell as well as NK cell mechanisms. What is not

clear, however, is how innate and acquired immune aspects can determine severity and recurrence of infection.

2 AIMS

When I started my PhD studies we decided to investigate several aspects of the immune system, specifically:

1) We wanted to study NK cell receptor expression during active and inactive SLE, and during other chronic inflammatory diseases, to better understand if NK cells can influence SLE pathology or can be affected by the disease (paper I).
2) We wanted to understand NK cell interaction with HSV1 infected cells using the knowledge from other herpes virus systems, and whether blood NK cells show phenotypic alterations during symptomatic viral reactivation (paper II).
3) We wanted to study the T-cell response during primary HSV1 and HSV2 genital infection and identify possible immune parameters predicting rate of recurrence following primary infection (paper III).
4) We wanted to study the innate and adaptive cell mediated responses during the asymptomatic phase in patients with recurrent HSV2 meningitis or recurrent genital manifestations. By comparing the two, and also by comparing them to the responses of healthy seropositives, we wanted to identify possible immunological deviations, innate or adaptive, that may be involved in the pathology of recurrent HSV2 meningitis (paper IV).

I will now discuss the results achieved during the last years.

3 RESULTS AND DISCUSSION

During the last years I have been involved in several projects, all directed to unravel immunological mechanisms implicated in chronic inflammatory or infectious disease.

3.1 PAPER I

In this paper we addressed whether NK cells show signs of alterations in SLE patients, which might indicate that they are involved in the pathogenesis of this disease. As noted in the introduction of this thesis, several data suggest the presence of defective NK cells in patients suffering from SLE. However, it had previously not been investigated whether these defects in NK cells occured pre or post disease development.

We analysed NK markers and function during different disease stages, i.e. during active disease and during remission, and included three distinct groups of controls: healthy controls, patients with rheumatoid arthritis and patients with IgA nephropathy, two autoimmune diseases characterized by organ specific chronic inflammation. We found a constant and specific increment of CD56 ^{bright} NK cells in SLE patients compared to the other control groups, and this was independent of disease activity. Increased frequencies of CD56 ^{bright} NK cells has not previously been observed in non-infectious diseases, but only in chronic infectious diseases such as HIV (180) and HCV (114).

We excluded the possibility that the observed increment in CD56 ^{bright} NK cells was a secondary effect caused by treatment, because even though all patients during active disease were under specific medication, patients during inactive disease were treatment-free. Different hypotheses emerged from our observation. The CD56 ^{bright} NK cells are normally more abundant in lymphoid tissues than in peripheral blood (21), due to an elevated expression of chemokines receptors such as CCR7 and CXCR3 (181) that facilitate their homing toward lymphoid tissue. The fact that we found increased level of CD56^{bright} NK in peripheral blood

of SLE patients might indicate a dysfunction in the homing capacity of this NK subset. Increased level of circulating CD56^{bright} NK might affect the cytokine profile detected in SLE patients, thus promoting disease progression.

Another factor that can influence the distribution of the two NK populations might be the oxidative stress observed in SLE patients. It has been shown that protein oxidation takes place during SLE due to the release of free oxygen radicals (182). A difference in survival in response of oxidative stress displayed by CD56 ^{bright} and CD56^{dim} NK cells can be an explanation for our observation. In fact it is known that CD56 ^{bright} are more resistant to cell death caused by oxidative stress than CD56 ^{dim} NK cells (183). This will simply cause an increase of CD56^{bright} versus CD56 ^{dim} NK cells in an environment more suitable for the first type of NK cells, and may be one explanation of our findings without any particular functional consequence. At sites of inflammation, such as in the inflamed joints, CD^{56bright} NK cells predominate (184). Also this may be due to a preferential survival in the oxidative milieu of inflammation, or may be a consequence of preferential recruitment of CD56^{bright} NK cells into inflammatory sites. Thus, blood NK cells in SLE are phenotypically comparable to the inflammatory synovial fluid NK cells in arthritis, possibly reflecting the systemic inflammatory condition of SLE.

We also have to acknowledge that the CD56 ^{bright} is considered to be an immature NK cell subset. Lately, a study from Romagnani et al (185) showed that peripheral CD56 ^{bright} NK cells can be induced to acquire the CD56 ^{dim} phenotype after cytokine stimulation. With this in mind, our results could indicate the accumulation of an immature phenotype of NK cells in SLE patients due perhaps to a defect that leads to an excessive production in the bone marrow or the lack of stimuli for NK cell maturation in the periphery

Another explanation would be to relate our results with published reports indicating an increased CD56 ^{bright} NK population after IFNβ treatment of multiple sclerosis (186) or after IL2 treatment during uveitis (187).

As already described previously, IFNα overproduction is a typical feature during SLE. Plasmacytoid DCs are the principal cell type responsible for this high production and this is elicited by immunocomplexes containing nucleic acids present in SLE patients (188). A high IFNα level has different effects on the immune response. It induces DC maturation (79), and this could then cause expansion of autoreactive T-cells. In vitro studies show that DCs in culture with sera from SLE patients are able to induce $CD8^+$ T-cell differentiation into CTLs with the potential to induce tissue damage (189). IFNα is also able to induce B cell activation, antibody production and Ig isotype switch (80, 190) (Fig6).

IFNα affects NK cells by inducing cytokine release and cytotoxic function (191), but there is no information about IFNα effects on the distribution of $CD56^{bright}$/$CD56^{dim}$ NK cell subsets. From our in vitro observation, we suggest that the observed $CD56^{bright}$ NK cell increment might be a consequence of the presence of type I IFN in patients, even if we did not find any correlation between IFNα serum levels and proportion of $CD56^{bright}$ NK cells. The increment of $CD56^{brigtht}$ NK cells that we could observe after in vitro IFNα stimulation was neither due to cell proliferation nor $CD56^{dim}$ NK cell death, and we conclude that it may be due to phenotypic changes of the $CD56^{dim}$ NK cell subtype, i.e. upregulated levels of CD56 and loss of CD16 induced by IFNα. The fact that IFNα serum levels did not correlate with the proportion of $CD56^{bright}$ NK cells does not exclude the possibility that the observed level of $CD56^{bright}$ NK cells during inactive disease phase is still due to IFNα. In fact, a recent paper, where the correlation between SLE disease activity and IFNα induced genes was studied (192), showed that IFN responsive genes are upregulated also during inactive disease. This implies that even though IFNα is not detectable in patient sera during disease remission, it may be produced in biologically relevant concentrations in inflamed tissue. Thus we speculate on a direct role of IFNα in elevating $CD56^{bright}$ NK cell proportions (fig 6).

Fig 6

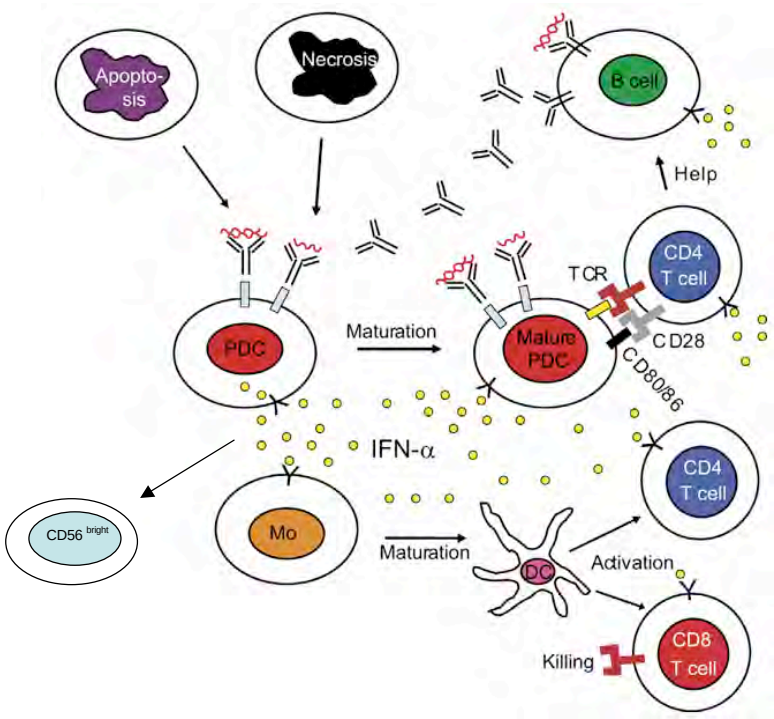

Fig.6: Schematic view of the IFNα network in SLE.
Possible roles for IFNα in increasing the frequency of CD56^{bright} NK cells in SLE patients
Modified from Rönnblom L and Pascual V, Lupus 2008;17(5):394-9

3.2 PAPER II

A number of publications on interactions between NK cells and different viruses in the Herpes family (148) led us to explore possible immune evasion mechanisms exerted by the α herpes virus HSV1. Herpes viruses have evolved several mechanisms to hide from the host immune system. The best characterised is HCMV, a member of the β herpes family. For example, HCMV interferes with MHC class I expression at different levels by direct downregulation of MHC class I molecule expression (193, 194) or peptide presentation (195) or even by coding for a MHC class I homologue, UL18 (196). According to the missing self-hypothesis, this should render CMV infected cells more susceptible to NK cells. However, CMV has in parallel also evolved the ability to escape NK cell recognition by means of several, almost overlapping mechanisms. The major pathway that is targeted by the virus is the NKG2D-NKG2D ligand recognition. NKG2D ligands are expressed upon cellular transformation or infection (197). There are at least seven different NKG2D ligands in humans (described in the introduction) and some present a high degree of genetic polymorphism (198). HCMV produces two proteins, UL142 and UL16, capable of dowregulating

surface expression and sequestering several NKG2D-ligands in the endoplasmatic reticulum (149-151, 199). UL142 is able to dowregulate only the full length MICA protein, i.e. not proteins encoded by truncated alleles of MICA (152, 199). The reason why there are so many ligands for one single receptor is still unknown. The most accredited hypothesis is that environmental pressure, such as viral infections, have imposed this high variation in order to allow some hosts to resist viral immune escape mechanisms. For example, among the 60 different MICA alleles, the most common in the Caucasian population is *008 that encodes a protein with a truncated transmembrane region (200). The cell surface expression of this variant of MICA is not downregulated by HCMV (199), and it may be that this allele has been selected for in evolution due to an advantage in protective immunity to HCMV.

We decided to undertake an in vitro study to see whether HSV1 had evolved similar mechanisms to escape NK cell surveillance. We analysed NKG2D ligand expression after HSV infection of an epithelial cell line, HeLa, and of a neural cell line, U373. We detected MICA surface dowregulation upon HSV1 infection of HeLa cells, while the total cellular MICA protein level remained constant. This finding suggests a similarity between HCMV and HSV. The MICA protein expressed in the cell line we used is encoded by *008 and truncated, thus it seems HSV uses a different mechanism than HCMV to achieve the same result. HSV2 infection has the same ability suggesting that the responsible gene could be conserved between HSV1 and HSV2 virus genomes. Although it cannot be excluded, it appears less likely that the two viruses possess unrelated genes to achieve the same effect. They share 83% sequence homology (201) and the majority of the sequence diversity is found in the genes coding for envelope proteins (202, 203). Using a viral gene deletion mutant, we excluded a role for ICP0, a gene known to promote NK cell recognition and killing of infected cells (136), in MICA downregulation. To better dissect which gene was involved in this phenomenon we questioned whether a late viral protein or gene is involved in this process by using a DNA polymerase inhibitor to block viral replication. With this method we were able to restore normal levels of MICA expression after infection, indicating that a late viral gene is involved in MICA dowregulation.

Does MICA dowregualtion affect NK cell recognition of infected cells? It was intriguing that despite this downregulation of MICA, the overall killing by NK cells of infected cells did not change compared to uninfected cells. This emphasized the complexity of NK-target cell interactions based on the integration of multiple signals. We speculate that the downregulation of inhibitory signals, MHC class I molecules, is neutralized by the downregulation of an activating signal, MICA surface protein. The net result of these two events on NK cell activation would thus be null. Hence, HSV can hide from T-cell recognition by downregulating MHC class I, and can counteract the NK cell activating effect this may convey by simultaneously downregulating MICA. Alternatively, or in addition, there might be ligands for other activating NK cell receptors that are induced by the virus.

As HSV infects epithelial cells as well as neural cells in vivo, we also investigated possible effects of NKG2D ligand expression on the U373 astroglioma cell line. Of the NKG2D ligands, uninfected HeLa cells expressed MICA only, while uninfected U373 cells expressed ULBP2. Interestingly, ULBP2 surface levels were downregulated by HSV1 infection, in analogy to MICA expression of HeLa cells. Thus, the virus can downregulate several NKG2D ligands, but leaves ligands to other activating NK cell receptors, such as Nectin and PVR, undisturbed.

We further investigated possible signs for NKG2D-NKG2D ligand interactions during HSV1 infection by analysing NK cell phenotype in peripheral blood from individuals suffering from recurrent HSV1 lip blisters. We detected a somewhat increased expression of NKG2D receptors, on NK cells exclusively, during the first two days after blister formation. Several factors can influence NKG2D expression. For example, oxidative stress observed during renal disease affects both NKG2D and MICA expression: NKG2D expression is reduced both on circulating NK and $CD8^+$ T-cells, while levels of membrane and soluble MICA are increased (204) . More over, it is known that membrane shedding of NKG2D ligands from tumor cells can reduce the expression of NKG2D on NK cells as well as on T-cells (205, 206) and NKG2D is downregulated in response to

increased or constitutive MICA expression, as observed in experimental studies (207). We found no evidence of involvement of soluble MICA in our study, since levels in serum were under the detection limit of our ELISA. NKG2D expression can also be induced by cytokines produced during viral infection, such as IL15. In accordance with other published data (141, 208), we observed an induction of NKG2D expression on NK cells cultured with IL15. We then studied the relationship between IL15 serum levels and NKG2D expression on NK cells, and found no correlation. IL15 is a very potent lymphocyte activator but detectable serum levels are seen only during chronic inflammation (209) as the cytokine is biologically active as a membrane bound form (210). A recent paper reported a tight interrelation between DCs and NK cells due to the capacity of TLR induced DC production and trans-presentation of IL15 to NK cells (211). In vitro, PBMC infection with HSV induces IL15 gene expression on monocytes (212). In this context, we can speculate that during productive HSV1 infection, membrane-bound IL15 on activated mucosal macrophages induces NK cells to upregulate NKG2D expression. These NK cells may thus have an increased potential to specifically recognize HSV1 infected, MHC class I low epithelial cells, despite lowered levels of MICA. The interpretations of our results so far discussed are based on the idea that the reactivation of the virus indirectly augments NKG2D expression. Another possibility is that a third factor (eg hormonal or stress related) predisposes both to increased NKG2D expression and reactivation of the virus.

In any case, NKG2D-NKG2D ligand interactions seem to have played a role in the evolution of HSV1 to adapt to its host, since the virus has the capacity to downregulate NKG2D ligand expression. Our results thus support a role for NKG2D in immune surveillance and recognition of HSV1.

3.3 PAPER III

The purpose of the next study was to analyse in more detail the immune response of subjects affected by HSV during the acute primary infection. We were

particularly interested in impaired or dysfunctional responses that might correlate with susceptibility to frequent or severe recurrences.

The first encounter with HSV1 infection normally occurs during adolescence, while HSV2 infection most often is acquired at the age of 20-30 years. Previously, viral encounter was earlier during lifetime, but acquisition has been delayed in the last two decades in many developed countries due to changes in life style. HSV2 seroprevalence in the population changes according with the age group analysed and differs also between countries. In Scandinavia, 15-35% of the adult population present HSV2 antibodies in the circulation (213). Infected subjects can transmit the virus in different ways. Sexual intercourse is the main transmission pathway, but other ways have to be considered. For example, vertical transmission from mother to child during birth that can cause disseminated infection or even neurological complications in the newborn (214). Immunocompromised hosts are at risk for frequent, persistent HSV infections (215). The course of infection in subjects without known immunodeficiency is variable. Almost 60% never experience clinical symptoms of their HSV2 infection (216). However, all infected subjects present intermittent asymptomatic viral shedding (217) and can thus transmit infection without themselves having symptoms. It has been shown that daily antiviral therapy using valacyclovir, can dramatically reduce the shedding and therefore the transmission risk (218).

Several reports indicate HSV2 as a risk factor for contracting HIV (219, 220), possibly due to the disruption of the genital epithelium during HSV2 that could favor HIV penetration and/or because HSV2 ulcerative lesions harbor high concentrations of activated CD4 lymphocytes (221), which are the target cells of HIV infection. In addition, HSV2 treatment in HIV co-infected patients has been shown to reduce HIV viral load and shedding in the genital area (222-224).

Upon HSV1 or HSV2 infection, the manifestation during the primary infection is often the most severe. Clinical symptoms such as mucocutaneous ulcerative and painful lesions, become milder over time, possibly due to a maturation of the memory compartment in the immune system (225). Among the individuals that

suffer from clinical HSV infection, the most common of which are oral or genital blisters, there is a big variation in number of recurrences, spanning from zero to twelveyear (226) and decreasing with time after primary infection (227). HSV reactivation is often preceded by a prodrome of pruritus, tingling, burning or swelling, indicating viral replication at sensory neuron terminals localized in the area of the future recrudescence. Previously, HSV1 was described as preferentially infecting orofacial epithelia while HSV2 was normally the cause of genital infections, but this typical picture is changing. In fact both viruses can infect either the oral or the genital area, and presently, HSV1 is as commonly the cause of genital infection as is HSV2 (228), which was also evident in our study . Clinical features of genital HSV1 cannot be differentiated from HSV2 infections; laboratory tests are necessary to identify the nature of the infection. What is different between the two viruses is the prognosis: genital HSV1 infection results in an lower average of recurrences during the first year compared to genital HSV2 infection, and some of the HSV1 patients will never experience any recurrences (229). This was also evident in our study. Both viruses can also cause neurological infection, for example encephalitis for HSV1 and aseptic meningitis for HSV2. The incidence of CNS manifestations is lower compared to genital infections (230) but it is probably underdiagnosed (personal communication by Franzén-Röhl E).

In the past years it became more clear that previous encounter with HSV1 does not prevent from HSV2 acquisition (231) and HSV1/HSV2 co-infection, has no beneficial effects in clinical outcome (232) compare to single infection. Thus, immune crossreactivity between HSV1 and HSV2 does not seem to translate into better protection from infection or higher ability to control viral reactivation.

We wanted to analyse whether anti-viral immune reactivity could determine whether an individual that first encounters the virus, will later on experience more or less recrudescences. The first challenge in this study was to properly classify the patients. Although none of the patients had experienced previous clinical symptoms, it became evident that some of them had had subclinical infections. By combining PCR on blister secretions or cerebrospinal fluid (CSF)

with serology, patients were divided into true primary infections, either HSV1 or HSV2, and "first episode" infections of the different sub-types. The latter were defined as first clinical eruption due to viral reactivation of a symptom-free latent infection. The PCR based disagnostic technique has a higher sensitivity (233, 234) and it was preferred to virus culture because virus is isolated from lesions in 80% of the primary infection and only in 25-50% of recurrent infections, and the percentage is even lower if the healing process has started (235). We used four different ELISA based systems in order to distinguish between primary infection, defined as initial absence of HSV antibodies at onset of clinical symptoms and subsequent development of HSV1 and HSV2 type-common, as well as HSV1 or HSV2 type specific, IgG antibodies over one year. Non-primary first episode infection was in most cases defined by the presence of HSV IgG antibody at first visit. During the classification we had to consider that HSV seroconversion, as detected by the type-common ELISA, should take place at the latest two weeks after onset of symptoms (236). One of the patients was included two weeks after the beginning of clinical manifestations and presented positive in serological test to HSV. She was included in the primary infection group because her HSV-specific cell-mediated immune (CMI) responses were comparable to those having primary infection. We followed the patients for a period of one year to be able to assess how many recurrences they were experiencing and when the seroconversion took place. The first observation, in accordance with the literature, was that HSV1 genital infection causes fewer recurrences than HSV2 infection (229, 235).

We collected three blood samples from each patient and we analyzed innate as well as adaptive immune responses. From previous studies, we know that reduced numbers or deficiency of NK and $CD4^+$ T-cells (107, 237) can lead to disseminated HSV infections. We thus characterized NK cells from patients and determined that there was no difference in NK cell numbers or phenotype over time in all three groups analysed. Neither could we detect abnormal NK cell IFNγ production nor cytotoxic activity tested against a standard tumor cell line lacking MHC class I molecules, K562. Therefore, our data argue against profound changes in NK cell phenotype or activity that could determine number of

recurrences in our patients, and ongoing infection did not seem to affect NK cell function or phenotype. However we cannot exclude that NK cells might present some difference in some other aspect that we did not study, such as granule release, TLR functions and KIR expression.

We detected elevated T-cell responses in primary infection compared to at month 2 and 12 thereafter, measured as $CD4^+$ T cell blast formation and cytokine secretion in whole blood cultures stimulated with HSV specific antigens. The elevated T cell responses might be an effect of ongoing infection due to the presence of active clonally expanded T-cells, while lower responses at month 2 and 12 may reflect a T-cell clonal contraction during latent periods of infection. One of the chemokines analysed presented a different pattern, IP10. IP10 is an IFN type I inducible chemokine that mediates the recruitment of NK and T-cells and it has been found in skin lesions of different diseases, such as SLE (238). It is involved in HSV infection as described for murine models, where IP10 deficient mice present reduced immune cell recruitment to sites of infection, higher viral load in CNS and higher mortality rate compare to wild type mice (239). The role of IP10 in our system would need more investigation but we can hypothesise that the increased production we detect during the one-year after the primary infection may be one cause of the observed reduction of the other cytokines. Thus, increased IP10 levels could lead to increased cellular infiltration and better immune control of viral reactivation. The increase in IP10 responses may be one explanation for the decrease with time in frequency of reactivation.

A clear linear correlation between the level of cytokine produced at first visit and number of recurrences experienced over one year was observed: the more cytokine produced, the less recurrences, in particular for IL4, IL10 and MIP1β. IL10 has been described as a regulatory cytokine able to reduce immune-pathological effects caused by excessive immune activation. It has been shown that during HSV1 infection IL10 is produced by T_{reg} cells and it reduces the production of IFNγ as well as IL2, hence limiting tissue damage (240). IL4 is able to induce differentiation of naïve T-cells into Th2 cells and also B cell class switching, and is considered an anti-inflammatory cytokine. IL4 and IL10 have

been described to be highly secreted in other chronic diseases such as allergy, HIV and also respiratory syncytial virus infection (241, 242), while specific antigen stimulation of PBMC from HSV2 symptomatic patients induces low levels of IL4 and IL10 (232). It is possible that these two antiinflammatory cytokines may reduce clinical symptoms due to their ability to limit the immune response, hence they may reduce pathology of HSV2 reactivation during the normal intermittent viral shedding that all infected subjects experience (217). Also MIP1β production correlated to lower number of clinical recurrences following primary infection. MIP1β is a chemokine which can be secreted by T and NK cells, and has been shown to have direct antiviral capacity by binding to gB protein on virions and generating pores in the viral envelope (243). Thus, MIP1β might be important in the elimination of virus both by recruiting other leukocytes and by directly killing virions. An efficient MIP1β production can result in limitation of the epithelial surface affected by viral infection thus limit production of virus particles that can escape and establish latency, consequently reducing the number of future recurrences.

3.4 PAPER IV

We proceeded with our investigation on herpes infections, this time seeking for immune dysfunctions that might correlate with one of the severe manifestations of HSV2, recurrent meningitis.

Aseptic meningitis is rarely seen upon HSV1 infection while it is the most common complication of HSV2 infection (244). HSV2 meningitis was first described in the context of Mollaret syndrome (Mollaret P "La meningite endothelio-leucocytaire multirecurrente benigne. Syndrome nouveau ou maladie nouvelle. Documents clinique" Rev Neurol Paris 1944) which includes all kinds of recurrent aseptic meningitis with symptom-free periods and presence of large and fragile endothelial cells in the CFS, the so called "mollaret's cells" (245). Mollaret himself suggested that a virus might cause the disease. Alphaherpes-viruses were ideal candidates because they are neurotropic, establish latency and may cause recurrent disease. Benign recurrent lymphocytic

meningitis following herpes meningitis has since then been described by several authors (246-249) and HSV2 is shown to be the major cause of benign recurrent lymphocytic meningitis (250-252). It has been determined that HSV2, together with enteroviruses, is the most frequent cause of aseptic meningitis (253, 254). It appears with or, more often, without mucocutaneous lesions.

The first episode of HSV2 meningitis can be experienced within two weeks from the primary genital infection (249) but can also appear without preceding mucocutaneous lesions. Recurrences, instead, are more often seen without any genital blister formation. During the acute phase, patients will experience headache, neck stiffness and in many cases fever. Females are more frequently affected than males, and this is also true for genital infections (225, 234, 252, 255). It can be that the female hormone cycle plays a role in the recurrences, there is a known association between herpes virus reactivation and the menstrual cycle (256). One suggested explanation has been that the anatomically larger area exposed and a larger viral load forwarded to the sacral ganglia in female are a reason for this. It should be noticed however that the frequency of asymptomatic individuals among seropositive subjects is higher in males than in females (226). The reason for this is unknown.

In mouse models of herpes HSV2 meningitis, the chemokines CCR5, CXCL9 and CXCL10 have been shown to be essential to promote cell trafficking and reduce viral load in the CSF (239, 257). Some evidence of human genetic variation influencing the incidence of recurrent meningitis has been reported. One paper shows association between recurrent HSV2 meningitis and polymorphisms in two genes: the IFNγ receptor and the mannose binding lectin (MBL) (258) but the authors did not study functional differences in the identified gene variants. Two other reports hypothesised that a low humoral immune response would be involved in the pathogenesis of recurrence (259, 260) possibly due to viral immune evasion mechanisms. To conclude, the current knowledge on immune responses in subjects affected by recurrent HSV2 meningitis is limited.

We thus hypothesised that an impaired cellular immune response would lead to a reduced ability to control viral spread and/or reactivation, and that this could be a possible explanation for recurrent meningitis (RM). We studied innate and adaptive immunity of subjects suffering from HSV2 recurrent meningitis. All patients had at least three prior admissions with confirmed HSV2 meningitis by PCR. We compared this group with subjects affected by recurrent genital infections (RG), and with healthy controls, either with detectable HSV-antibodies or seronegative to HSV. The patients were included in the study when they had no symptoms of ongoing HSV or other infections. We did not detect any abnormality in lymphocyte cell numbers in the RM patients, and T and NK cell phenotypes were comparable to controls. As T-cells have been implied in controlling viral reactivation, the major question was whether T-cell responses to HSV specific stimulation were impaired in our recurrent meningitis patients.

We analysed $CD4^+$ T-cell blast formation as well as cytokine and chemokine production and, contrary to our expectation, we found significantly elevated T-cell responses to antigen-specific stimulation in RM patients compared to all other groups. The cytokines induced by antigen activation was very broad, spanning from Th1 to Th2 and Th17 type cytokines. When comparing RM and RG versus healthy control we found that while both groups were able to produce higher levels of Th1 cytokines such as IL12 and TNFα and also IL10, when stimulated with HSV nuclear lysate, the RM group specifically responded with high levels of Th2 cytokines such as IL4 and IL13, and the same trend was observed for IL5. These cytokines have been involved in allergic responses but lately it has been reported that IL4 and IL13 can have an important role in regulation of tight junctions in the mucosal epithelia in HIV (261) and ulcerative colitis (262). Moreover, FACS analysis revealed higher expression of TLR3 and TLR9 in DCs from RM patients compared to healthy seropositive donors. IFNα production upon stimulation using TLR3 agonists, but not agonists to TLR4, 7 or 9, was augmented in the RM patients. Thus, we observed increased T-cell responses as well as increased TLR expression and function.

The regulation of TLR expression is not fully understood. The presence of pathogens as well as cytokines such as IFNγ, normally released during infections, can induce TLR expression (263). There is some evidence that the expression levels of TLRs are increased during HIV viral infection (264) as well as during inflammation (265). In the context of HSV infection, the Us3 gene from HSV1 has been reported to reduce TLR3 mRNA levels (266), while TLR2 and 4 surface levels are reduced upon HSV1 infection of monocytes in vitro (267).

We also observed a somehow increased production of IFNγ from NK cells when stimulated with live HSV2. Recently, the presence of memory in the NK cell compartment has been suggested in murine CMV infection. "Virus experienced" NK cells were able to more efficiently produce IFNγ in response to viral infection compared to "naïve" NK cells (268). Similar to this finding, I speculate that our observation could represent a virally induced augmentation of NK cell long-term responses in humans.

Taken together, our data describe that rather than a low anti-viral immune response, RM patients present an elevated ability to respond to HSV stimulation, with respect to innate immunity as well as specific adaptive immunity. The question now is if what we observed is merely a consequence of the disease or if it may be related to the cause. One could speculate that the increased immune responses seen in RM simply reflect repeated and/or high dose exposure to antigen and virus during recurrences. The cause of the initial and recurrent meningitis would in this scenario not be a stable impairment in immune responses, but something else (eg local vulnerability of CNS tissues, temporary immune impairment, and exposure to unusually high virus dose in primary infection). Thus, enhancing the immune responses during recurrences would facilitate viral control and decrease meningitis symptoms. On the other hand, it might be that the elevated immune response is the cause for the different clinical outcome, ie viral spread to CNS rather than retrograde transport of virus followed by genital infection. In this scenario, elevated T-cell responses, possibly due to elevated innate immune responses, promote spread to the CNS, for example by enhancing inflammation of the neural ganglia with consequent meningitis.

We do not have further evidence for any of these scenarios. However, our data certainly speak against permanently impaired immune responses being the cause for RM. Several reports corroborate this: they describe disseminated HSV skin infections in immune-compromised subjects, including HSV2 genitalis, but there is no evidence for enhanced meningitis associated with immune suppression (269, 270).

Due to observations in paper III we speculate that the pathogenesis of RG and RM might differ because of the different cytokine profiles they elicit. Thus, the elevated immune responses observed in RG patients is not the cause of the recurrences, since we found in paper III that initial high cellular mediate immune responses predict low recurrence incidence. We instead speculate that the observed difference in cytokine profile between the two groups, ie IL4 and IL13, might be the crucial mechanism behind the different clinical outcomes. In this scenario the increased level of these cytokines would lead to alteration in the epithelial mucosa of the genital area, and perhaps also in the interface between the ganglia and the cerebro spinal fluid, hence facilitating viral spread in the CNS with consequent meningitis. Testing in a prospective study whether elevated immune responses during primary infection, in particular IL4 and IL13, predisposes to RM would require a very high number of patients, since the incidence of RM is low. Regarding genital manifestation, our observations in paper IV suggest that the low specific T-cell response in primary HSV2 infection in subjects who later develop frequent recurrencies (paper III) change with time. The patients with RG tested in asymptomatic phases in paper IV also had elevated responses, suggesting that such patients eventually develop strong responses.

In conclusion, we have demonstrated for the first time that patients with recurrent HSV2 meningitis show enhanced specific T-cell responses to HSV antigens, with a unique Th2 profile, and also somewhat enhanced TLR3 mediated responses. This was contrary to our starting hypothesis. It cannot be excluded that hyper immune responses can contribute to the pathogenesis of recurrent meningitis. It is

also possible that the increased immune responses is instead induced during repeated presentations and activations of the immune system during recurrences, which themselves are consequences of another mechanism that remains to be shown.

4 CONCLUDING REMARKS

In this thesis I studied some aspects of the innate and adaptive compartments of the immune system in SLE and more extensively in herpes infections.

In **paper I** we determined that in subjects affected by SLE the proportion of $CD56^{bright}$ NK cells is significantly increased, and this phenomenon is not dependent on disease activity. We also speculated that it might be due to increased levels of IFNα at sites of inflammation, a typical hallmark of SLE patients. Further studies of IFNα induced gene profiles related to blood NK cells of patients with inactive SLE might highlight possible connections between our observations and disease pathology.

From **paper II** we postulate a possible role for the activating NK cell receptor NKG2D in the immune response against HSV1 infection. It would be interesting to determine whether the observation of higher expression of NKG2D on blood NK cells during the acute phase of reactivation has consequences on the capacity of NK cells to kill HSV1 infected epithelial cells. More over, it would be interesting to study NK cells at the site of infection, possibly by studying extensive oro-mucosal infection, for example during childhood.

From **paper III** the described negative association between strength of the specific T-cell response during primary HSV infection and the frequency of clinically documented recurrences, could have several interpretations. One interpretation is that a strong inherent ability to respond to HSV virus reduces the viral load available to establish latency, and that the frequency of recurrences depend on the initially established viral dose. Another possibility, that does not exclude the first, is that the strong initial response also reflects the capacity for immune surveillance against recurrence during latency. Finally, it is also possible that there may be no causal relationship between initial immune response and frequency of recurrences; both might be influenced by a third, unknown factor. Considering the results from paper IV where recurrent genitalis patients show

high cellular immune responses compare to healthy subjects, our results suggest that an initial high immune response will contribute to reduced recurrences while subjects affected by recurrences will show elevated immune responses as a result of constant viral reactivation. To understand the role of immune responses in deciding symptomatic versus asymptomatic viral reactivation, it would be interesting to study adaptive and innate immunity in individual RG patients in repeated blood samples. In vitro immune responses would then be compared between asymptomatic viral production and symptomatic blister formation, and correlated to viral load in genital secretions.

In **paper IV** we examined the immune response of patients affected by recurrent meningitis caused by HSV2 infection. We determined that these patients have elevated TLRs expression, elevated IFNα production to certain stimuli and elevated specific T-cell responses when compared to patients with recurrent genital infection and to healthy seropositive donors. The augmented responses were particularly impressive for the cytokines IL4 and IL13. One interpretation is that long term infection would lead to repeated viral presentation to the immune system and thus repeated immune activation that would simply translate in higher cellular responses, with higher viral load in RM compared to RG. Another possibility is that increased innate immune responses might be the cause for elevated T-cells responses, which in turn contribute to the pathogenesis of meningitis. To asses whether the immune responses is the cause for RM it would be of interest to plan a prospective study, similar to the one developed in paper III, but with focus on primary meningitis and see if their immune responses changes or not over time after establishment of recurrences. Furthermore, it would be interesting to perform epidemiological studies of immune compromised patients (transplanted patients on immune suppression as well as HIV infected patients) to understand whether RM is an immune mediated disease. If so, the incidence in these patient groups would be increased compared to in the general population.

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Here I am, writing the most terrifying part of this thesis, the part that EVERYONE reads, therefore the one that will get more comments.

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